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Peng, S ; Hummerjohann, J ; Stephan, Roger ; Hammer, P

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Interpretive Summary (100 words)

Heat resistance of *Escherichia coli* strains at different subpasteurization conditions

In this study, different thermisation conditions, a mild heat treatment of raw milk, reflecting commercial conditions are tested for their efficiency in reducing *Escherichia coli* strains in a pilot plant pasteurizer. Three of nine *E. coli* strains showed an increased thermotolerance.

Short communication:

Heat resistance of *Escherichia coli* strains in raw milk at different subpasteurization conditions

S. Peng,*† J. Hummerjohann,† R. Stephan,* and P. Hammer‡[1]

*Institute for Food Safety and Hygiene, University of Zurich, 8057 Zurich, Switzerland

†Agroscope Liebefeld-Posieux, 3003 Bern, Switzerland

‡Department of Safety and Quality of Milk and Fish Products, Max Rubner-Institute, Federal Research Institute of Nutrition and Food, 24103 Kiel, Germany

[1]Corresponding author: Philipp Hammer, Department of Safety and Quality of Milk and Fish Products, Max Rubner-Institute, Federal Research Institute of Nutrition and Food, Hermann-Weigmann-Strasse 1, 24103 Kiel, Germany, Phone +49 431 609 2430, Fax +49 431 609 2308, philipp.hammer@mri.bund.de

ABSTRACT

A commonly applied treatment of raw milk to reduce bacterial loads is the short-time application of heat at subpasteurization levels under continuous flow, generally referred to as thermisation, as this retains some of the beneficial properties of raw milk. In a previous study, *Escherichia coli* strains exhibiting an increased thermotolerance were found, demanding investigations on their ability to survive thermisation. Nine *E. coli* strains, including four Shiga toxin-producing *E. coli* (STEC) strains, were investigated for their reduction during thermisation treatment in raw milk using a pilot plant pasteurizer, to reflect typically applied commercial conditions.

Six of the nine *E. coli* strains, including the four STEC strains, were similarly inactivated at 60, 62.5 and 65°C, while for three *E. coli* strains an increased thermotolerance was observed. The reduction of all strains was below 2 $\log_{10}$ at 60 and 62.5°C within 25 s. At 65°C, six of nine *E. coli* strains were reduced for at least 5 $\log_{10}$ after 25 s while at 67.5°C, such a reduction was observed for eight strains. For the *E. coli* strain FAM21805 a much higher thermotolerance was found. It has to be considered that for some *E. coli* strains time-temperature combinations above 65°C were required to obtain a substantial reduction during a thermisation treatment.

Key words: thermisation, *Escherichia coli*, heat treatment, subpasteurization

48 The use of raw milk, e.g., for the production of cheese, offers natural enzymes and microflora
49 that give the final product a desired taste of special quality. However, the use of raw milk
50 constitutes also an increased risk for the survival or even multiplication of pathogens during
51 cheese making, including Shiga toxin-producing *Escherichia coli* (STEC) (Deschenes et al.,
52 1996; Espie et al., 2006; Peng et al., in-press).
53 Different methods are used to reduce bacterial loads in raw milk among which thermisation -
54 a heat treatment at subpasteurization temperatures for short time periods - constitutes a
55 relatively mild treatment that retains desired features beneficial for the taste of the product.
56 Thermisation reduces bacteria that potentially could produce proteases and lipases, which
57 may negatively affect cheese ripening. In addition, thermisation is frequently used by cheese
58 manufacturers in Switzerland and other countries to improve the safety of the products.
59 According to milk processing and quality management (Lewis and Deeth, 2009), 57-68°C are
60 applied for at least 15 s while the milk should remain phosphatase-positive (parameter to
61 distinguish it from pasteurized milk).
62 The level of *E. coli* rarely exceeds 10^4 CFU/ml in raw bulk tank milk and in raw commingled
63 silo milk (Van Kessel et al., 2004; Jackson et al., 2012). Therefore a reduction of 5 $\log_{10}$ will
64 inactivate at least the majority of *E. coli* possibly occurring in raw milk and was used as
65 guidance value for estimation of thermisation efficiency in this study (5-D criterion).
66 To characterize the reduction of bacteria due to heat treatments two parameters, *D*- and *z*-
67 values, are defined under the assumption that the reduction follows first-order kinetics. In this
68 study *D*- and *z*-values of nine *E. coli* strains isolated from raw milk cheese (Peng et al., 2012)
69 were determined using a pilot plant pasteurizer to apply thermisation treatment, which reflects
70 conditions used by cheese manufacturers. The pilot plant pasteurizer is constructed in a way
71 to reflect a commercial pasteurization plant in miniature (for details see: Hammer et al., 2002,
72 2005). Milk is heated in continuous flow, whereby two plate heat exchanger sections, heater
73 and cooler, are used. Basic technical data for the pasteurizer are as follows: A sample volume

74 up to 30 l can be processed at holding times of 2-60 s (holding time depends on flow rate and
75 holder volume). The flow rate can be varied between 15-80 l/h and a temperature range
76 between 40-145°C can be covered with an accuracy of ±0.2 l/h and ±0.2°C, respectively.
77 Reynolds numbers are dependent from holding time and holding section. With the holder
78 utilized in this study Reynolds numbers are approx. 1200 at 25 s holding time and approx.
79 2000 at 15 s holding time, which indicates flow at the transition between laminar and
80 turbulent flow (Kessler, 1981). Alterations in the flow rate are of influence on the residence
81 time distribution but can be neglected if the curve of the residence time distribution is
82 symmetrical. At Bodenstein number of > 640 a symmetrical residence time distribution can be
83 proposed (Rao and Loncin, 1974), which was the case in all conditions applied. At low flow
84 rates and low temperature, as applied in this study, the inactivation during heating and cooling
85 within the plate heat exchangers can be neglected compared to the inactivation within the
86 holding section.
87 Before each experiment the entire pilot plant was operated with water for 30 min at 98°C to
88 clean and decontaminate it. During operation the milk was continuously stirred. Temperature
89 and flow rates were adjusted at the registration board. In consideration of the total volume of
90 the pilot plant and the residence time distribution, after readjustments at least 1 l of milk
91 passed the plant before samples were collected to ensure that an appropriate amount of milk
92 heated at the target temperature had passed the sampling valve. Sampling was carried out with
93 sterile plastic syringes from a respective rubber stoppered valve located downstream of the
94 cooling section. After use the pilot plant was cleaned and disinfected by circulation of an
95 alkaline cleaner for 30 min at 80°C, an acidic cleaner for 20 min at 60°C and a final rinse with
96 water for 30 min at 98°C.
97 All *E. coli* strains used in this study (Table 1), were isolated from semi-hard raw milk cheeses
98 with the exception of strain FAM21805, which was isolated from a soft raw milk cheese, and
99 strain N09-1208, which was isolated from vat raw milk. Strains were maintained at -80°C in

100 the Microbank system (Pro-Lab diagnostics, Richmond Hill, Canada). For precultivation, one
101 bead was used to inoculate 10 ml of trypticase soy broth (TSB; Oxoid, Wesel, Germany) and
102 incubated at 37°C for 24 h. Subsequently 1 ml of this preculture was used to inoculate 500 ml
103 of fresh TSB, three Erlenmeyer flasks in total for each experiment. The flasks were incubated
104 on a shaker (60 rpm) for 24 h at 37°C.
105 For the experiments the bacteria were harvested by centrifugation for 10 min at 6,500 x g at
106 22°C. Pellets from three Erlenmeyer flasks were resuspended in a small amount of raw milk
107 and added to 30 l of milk for the heating experiments. Prior to the experiment the milk was
108 stirred for at least 5 min to ensure proper homogenization and stirring was continued during
109 the entire processing. The milk used for this study was whole raw milk, obtained from the
110 MRI`s research farm. It was collected with sterilized milking equipment after thorough teat
111 cleaning from selected cows to obtain a bacterial count as low as possible. Total colony
112 counts below 200 CFU/ml were achieved with this procedure.
113 To count the viable bacteria after heating, samples were immediately serially diluted 1:10 v/v
114 with peptone saline solution (1 g tryptone from caseine peptone [Oxoid] supplemented with
115 8.5 g/l sodium chloride [Merck, Darmstadt, Germany]). From the appropriate dilutions, 0.1 ml
116 in duplicates were spread onto blood agar plates (Columbia-agar supplemented with 5% (v/v)
117 sheep blood [Oxoid]), and incubated at 37°C for 72 h.
118 In pre-trials strains were tested at heating temperatures of 57.5, 60, 62.5, 65, 67.5 and 70°C, at
119 a holding time of 20 s to determine the temperature region where considerable reduction can
120 be expected (data not shown). For the experiments to determine the *D*-values and z-values the
121 strains were heat treated at three successive of the preselected temperatures and at holding
122 times of 15, 20 and 25 s utilizing one inoculated 30 l sample of milk for each run of the pilot
123 plant. The holding times correspond to flow rates of 20.3, 25.2, and 33.7 l/h, respectively.
124 Temperature steps of 2.5°C and time gaps of 5 s were chosen. The activity of alkaline
125 phosphatase (ALP) in the heat-treated milk was analyzed according to ISO 11816-1:2006

126 (ISO, 2006). After 25 s heat treatment with all temperatures applied ALP activity in the milk
127 was higher than the threshold level for pasteurized cow's milk (350 mU/l; data not shown).
128 For the calculation of *D*-values $\log_{10} N_t$ (count of surviving bacteria) divided by $\log_{10} N_0$
129 (initial count) was plotted against time. A log-linear regression curve was calculated utilizing
130 Microsoft Excel for Mac 2011. The *D*-value was calculated as the divisor of 1 and the slope
131 of the regression curve. Coefficient of determination (R squared) was calculated for analysis
132 of the fit of the model. *D*-values observed from three experiments were averaged and standard
133 deviation was calculated. To determine *z*-values the averaged *D*-values were $\log_{10}$
134 transformed, plotted against temperature and the same procedure as described above was
135 applied.
136 The heat resistance parameters (*D*- and *z*-values) of the nine *E. coli* strains are summarized in
137 Table 1. According to Martens (2003), the fit of the log-linear model was high as the
138 coefficient of determination (R^2) was at least 0.7 for 75 and 0.9 for 51 of the 81 calculated *D*-
139 values. Five additional *D*-values ($R^2 \geq 0.6$) were used for further evaluation while data of one
140 measurement was discarded because of its low coefficient of determination (R^2 0.3).
141 The measurement inaccuracy of the pilot plant pasteurizer and especially the low slopes of
142 regression curves for higher *D*-values are attributed to cause the larger standard deviations of
143 the higher *D*-values. Therefore the calculated *z*-values are a reference point that should be
144 carefully regarded. This is exemplified by the observation that the *z*-value of the least
145 thermotolerant strain FAM21846 is higher than for the other strains, including the non-
146 pathogenic *E. coli* strains exhibiting increased thermotolerance. On average of the nine *E. coli*
147 strains a *z*-value of 4.3 ± 0.9°C was observed, which is consistent with the data collected by
148 Soerqvist (2003). Nevertheless, literature data for *D*- and *z*-values of *E. coli* show
149 considerable variation due to differences in the experimental design (e.g. food matrix, culture
150 media; van Asselt and Zwietering, 2006).

151 Six of the nine *E. coli* strains, including the four STEC strains, showed a similar inactivation
152 at 60, 62.5 and 65°C. At 60 and 62.5°C, the *D*-values ranged from 12.7 to 80.5 s, resulting in
153 a decrease of at most 2 $\log_{10}$ due to thermisation within 25 s, while at 65°C *D*-values were
154 below 5 s, which corresponds to a reduction of more than 5-D within 25 s thermisation. This
155 observation is in agreement with the findings of Schlesser et al. (2006), who proposed a
156 subpasteurization heat treatment of milk at 64.4°C for 17.5 s before using the milk for
157 Cheddar cheese production based on a demand of a 5-D reduction of *E. coli*.
158 For three *E. coli* strains, however, a much increased thermotolerance was observed. Unlike
159 the other six *E. coli* strains, these three strains were not reduced more than 2 $\log_{10}$ within 25 s
160 thermisation at 65°C. At 67.5°C, a 5-D reduction was observed within 25 s for the strains
161 FAM19195 and FAM21843. Nevertheless, strain FAM21805 exhibited a $D_{67.5}$ of 47.1+/-8.1 s
162 and therefore was barely reduced due to thermisation at 67.5°C. At 70°C this strain finally
163 exhibited a stronger reduction and the 5-D criterion was achieved within 25 s thermisation.
164 This observation shows that some *E. coli* strains may even survive a thermisation treatment at
165 65 or 67.5°C. The mechanisms, which increase the thermotolerance of these strains, remain to
166 be further investigated. The four STEC strains that were used in this study do not shown an
167 increased thermotolerance.
168

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Table 1. *D*-values (s) and *z*-values (°C) of nine *Escherichia coli* strains. Average and standard deviation of three experiments per strain and temperature for calculation of *D*-values. The *z*-value was calculated from the average *D*-values

		Temperature (°C)					
Strain	Serotype	60	62.5	65	67.5	70	z value
FAM21846	O16:H21	21.9 ± 4.9	12.7 ± 2.2	3.3 ± 0.1	nd[3]	nd	6.1
K133[+]	O113:H4	48.4 ± 22.0	49.7 ± 19.9	4.6 ± 0.9	nd	nd	4.9
N09-1208[+]	O26:H11	49.8 ± 16.4	31.9, 32.3[2]	3.4 ± 0.6	nd	nd	4.3
K303	O9:[H21][1]	53.8 ± 8.7	29.4 ± 7.1	2.9 ± 0.3	nd	nd	4.0
K331/4[+]	O91:H21	71.7 ± 26.8	23.6 ± 12.4	3.2 ± 0.4	nd	nd	3.7
K356[+]	O2:H27	80.5 ± 26.0	47.9 ± 7.7	3.0 ± 0.2	nd	nd	3.5
FAM19195	O8:H21	*	131.4 ± 51.3	17.4 ± 3.9	4.5 ± 0.4	nd	3.4
FAM21843	O178:H12	*	*	27.1 ± 6.5	3.2 ± 0.3	2.8 ± 0.2	5.1
FAM21805	O68:H14	*	*	93.4 ± 47.0	47.1 ± 8.1	4.2 ± 0.4	3.7

[+]Shiga toxin-producing *E. coli* strain

[*]no significant reduction observed (reduction ≤ 0.25 $\log_{10}$).

[1]phenotypically non-motile.

[2]only two experiments due to low coefficient of determination.

[3]not determined.